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Possible mechanisms regulating ATP- and thimerosal-induced Ca^{2+} oscillations in the HSY salivary duct cell line

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Abstract

The ATP-induced oscillatory changes in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) were analysed in HSY cells, a salivary ductal cell line from human parotid, using a fluorescence ratio imaging system. At concentrations higher than 1 μM, ATP caused sinusoidal $[Ca^{2+}]_i$ oscillations due to the periodic release and reuptake of Ca^{2+} by intracellular Ca^{2+} stores. The phorbol ester 4β-phorbol 12,13-dibutyrate (PDBu) changed the $[Ca^{2+}]_i$ oscillations to a single spike. The inhibitory effect of PDBu on the $[Ca^{2+}]_i$ signals was reversed by protein kinase C (PKC) inhibitors such as staurosporine and chelerythrine chloride. However, preincubation of the cells with the PKC inhibitors did not affect the pattern of the ATP-induced $[Ca^{2+}]_i$ oscillations. The desensitization of the $[Ca^{2+}]_i$ response observed during prolonged stimulation with ATP was also not prevented by the PKC inhibitors. Incubation of HSY cells with the sulphydryl reagent thimerosal, which enhances the sensitivity of inositol 1,4,5-trisphosphate (IP_3) receptors, caused repetitive Ca^{2+} release from intracellular Ca^{2+} stores resulting in baseline spikes of $[Ca^{2+}]_i$. The thimerosal-induced $[Ca^{2+}]_i$ oscillations did not change in the presence of PDBu and the phospholipase C inhibitor U73122. Thus, we could not provide evidence that negative feedback by PKC plays a central role in the regulation of ATP-induced $[Ca^{2+}]_i$ oscillations. These results suggest that the $[Ca^{2+}]_i$ oscillations, at least the baseline spikes, in HSY cells can be generated without stimulating the formation of IP_3.

Keywords: Ca^{2+} oscillation; Protein kinase C; IP_3 receptor; Thimerosal; HSY cell

1. Introduction

In a variety of cell types, stimulation of surface membrane receptors linked to the phosphoinositide signalling pathway often results in oscillatory changes in cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). Although different models have been proposed to explain the generation of such $[Ca^{2+}]_i$ oscillations [1–4], the molecular mechanisms are not fully established. Based on the findings that activators or inhibitors of PKC prevent or modulate $[Ca^{2+}]_i$ oscillations [5–8], one of the models postulates that protein kinase C (PKC) plays the key role in the mechanisms regulating $[Ca^{2+}]_i$ oscillations. In this model, the $[Ca^{2+}]_i$ oscillations reflect an oscillatory formation of inositol 1,4,5-trisphosphate (IP_3) resulting from negative feedback by PKC on IP_3 formation. However, the involvement of PKC in $[Ca^{2+}]_i$ oscillations is still the subject of debate. In several models,

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it has been suggested that the feedback by PKC and the fluctuations of IP_3 are not essential for the regulation of $[Ca^{2+}]_i$ oscillations. There the Ca^{2+}-induced Ca^{2+} release and the positive and negative feedback effects of Ca^{2+} itself on IP_3 receptor activity are thought to play an important role in the generation of $[Ca^{2+}]_i$ oscillations [9–13].

The HSY cells, a salivary ductal cell line from human parotid, have been used as a model system for the study of intracellular Ca^{2+} signalling events [14–17]. Tanimura and Turner [15] have found that application of IP_3 to saponin-permeabilized HSY cells can cause repetitive release and reuptake of Ca^{2+} by intracellular stores, supporting the model that the $[Ca^{2+}]_i$ oscillations arise directly from the feedback effects of Ca^{2+} itself on IP_3 receptor activity.

ATP is a potent agonist to mobilize Ca^{2+} from intracellular stores by the activation of phosphoinositide hydrolysis in salivary ductal cell lines [18]. To assess the involvement of PKC in ATP-induced $[Ca^{2+}]_i$ oscillations, the present study has examined the effects of PKC modulators on the $[Ca^{2+}]_i$ oscillations in HSY cells. Further, we have analysed the changes in $[Ca^{2+}]_i$ during treatment with the sulphydryl reagent thimerosal, which can sensitize cells to basal IP_3 levels [19,20]. As the sulphydryl reagents are able to induce a Ca^{2+} release from IP_3 receptor channels without stimulating IP_3 formation, analysis of the thimerosal-induced Ca^{2+} release may provide information when considering the mechanism regulating $[Ca^{2+}]_i$ oscillations in HSY cells.

2. Materials and methods

2.1. Materials

Fura 2-acetoxymethyl ester (fura 2-AM) was purchased from Dojin Laboratories (Kumamoto, Japan). The ATP, chelerythrine chloride and 4α-phorbol 12,13-didecanoate (4α-PDD) were purchased from Sigma (St. Louis, MO, USA). Thimerosal was from ICN Biomedicals (Aurora, CO, USA). Staurosporine, thapsigargin, 4β-phorbol 12,13-dibutyrate (PDBu), U73122, and U73343 were from Wako Pure Chemical (Osaka, Japan). K-252a was from Kyowa Medex (Tokyo, Japan).

2.2. Cell culture

HSY cells were grown as described elsewhere [14] in a 50:50 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium supplemented with 10% newborn calf serum, 2 mM glutamine, 100 μg/ml penicillin and 100 μg/ml streptomycin. For the fluorescence experiments, the HSY cells were transferred from plastic culture dishes to 13×13 mm sample chambers consisting of cylinders glued to round glass coverslips, and allowed to grow for a further 2–4 days before use.

2.3. $[Ca^{2+}]_i$ measurement

HSY cells in sample chambers were washed with HBSS-H (Hanks' balanced salt solution buffered with 20 mM HEPES-NaOH (pH 7.4)) and then incubated with 2 μM fura 2-AM for 40–60 min at room temperature. After loading with fura 2, the chamber was rinsed twice with HBSS-H and mounted on the stage of a Nikon Diaphot inverted microscope (Nikon, Tokyo, Japan) equipped with a Nikon CF Fluor 20× objective. Excitation wavelengths were set at 340 and 380 nm. Emitted light was passed through a 400 nm dichroic mirror, filtered at 520 nm and collected by a cooled CCD camera (Hamamatsu Photonics, Shizuoka, Japan). The fluorescence ratio images were digitized at 5 s intervals and stored in an ARGUS HiSCA imaging system (Hamamatsu Photonics). The changes in $[Ca^{2+}]_i$ were shown as the 340/380 nm fluorescence ratio.

The vacuum line was placed in the sample chamber and adjusted so that the volume of the solution was maintained at approx. 50 μl. Solution changes were accomplished by the addition of 500 μl of fresh solution to the chamber. All experiments were carried out at room temperature.

3. Results

3.1. Characterization of ATP-induced $[Ca^{2+}]_i$ oscillations

HSY cells were intermittently exposed to successively greater concentrations of ATP. Fig. 1A shows

typical changes in $[Ca^{2+}]_i$ analysed by single-cell Ca^{2+} recording. In most cells, stimulation with low concentrations of ATP (≤ 0.5 μM) caused only a single spike in $[Ca^{2+}]_i$ which did not result in oscillatory responses. At concentrations higher than 1 μM, ATP induced sinusoidal oscillatory changes in $[Ca^{2+}]_i$ in 118 out of 131 cells examined. The number of oscillatory peaks observed during the 100 s stimulation increased in a concentration-dependent manner, and stimulation with a supramaximal concentration (100 μM) of ATP resulted in a plateau-like increase in $[Ca^{2+}]_i$ with little or no oscillations. Removal of extracellular Ca^{2+} by switching to Ca^{2+}-free medium containing 0.2 mM EGTA did not alter the oscillatory pattern of $[Ca^{2+}]_i$ (Fig. 1B), indicating that the ATP-induced $[Ca^{2+}]_i$ oscillations are primarily dependent on the periodic release and reuptake of Ca^{2+} at intracellular Ca^{2+} stores.

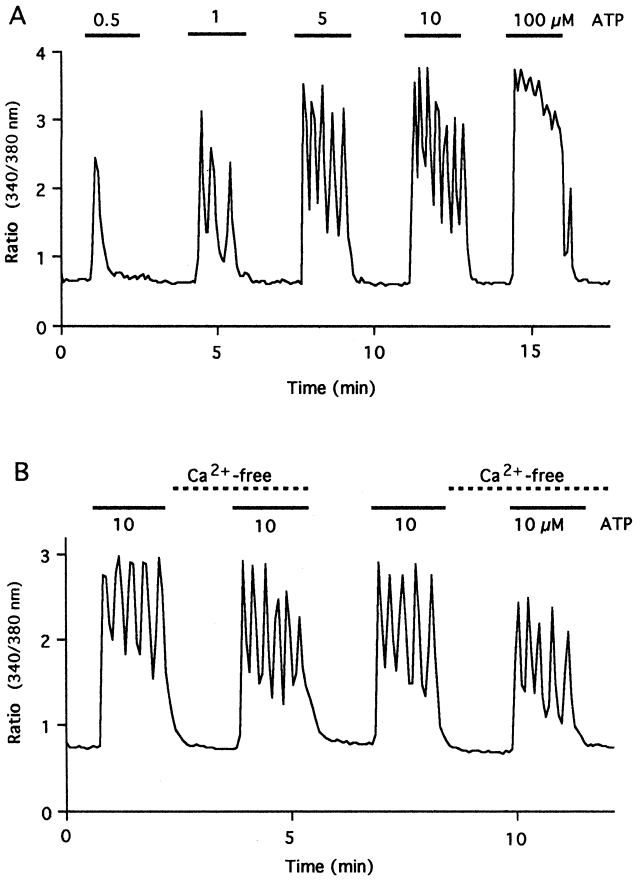

Fig. 1. ATP-induced $[Ca^{2+}]_i$ oscillations in single HSY cells. The $[Ca^{2+}]_i$ was measured in single HSY cells loaded with fura 2 and is shown as the fluorescence ratio of 340/380 nm. ATP was applied during the period indicated by the solid horizontal bars. (A) Changes in $[Ca^{2+}]_i$ recorded from a single cell exposed intermittently to increasing concentrations of ATP. The response shown is representative of more than 100 cells. (B) Changes in $[Ca^{2+}]_i$ in a single cell exposed repeatedly to 10 μM ATP in Ca^{2+}-free and Ca^{2+}-containing external solution. The external solution was exchanged to Ca^{2+}-free solution containing 0.2 mM EGTA as indicated by the dotted line.

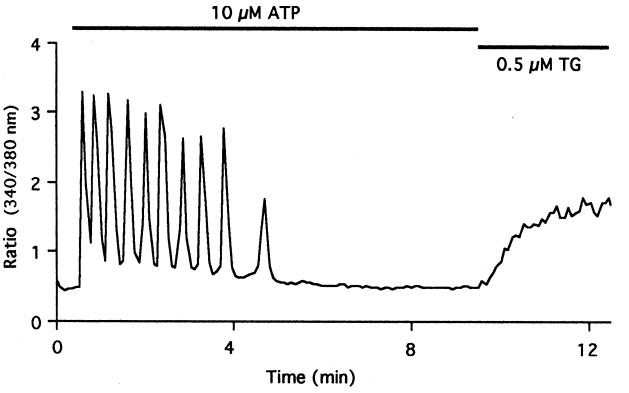

Fig. 2. Desensitization of the $[Ca^{2+}]_i$ response during prolonged ATP stimulation. Cells were exposed to 10 μM ATP and subsequently to 0.5 μM thapsigargin (TG) during the period indicated by the horizontal bars.

Fig. 2 shows a typical oscillatory pattern in $[Ca^{2+}]_i$ in a single HSY cell exposed continuously to 10 μM ATP. The ATP-induced oscillatory response was gradually attenuated during the stimulation and ceased within 10 min after stimulation. This cessation cannot be attributed to depletion of intracellular Ca^{2+} stores, since subsequent addition of 0.5 μM thapsigargin (TG), an inhibitor of the endoplasmic reticulum Ca^{2+} pump, caused a further increase in $[Ca^{2+}]_i$ (Fig. 2). Prolonged stimulation with lower concentrations (0.5 or 1 μM) of ATP resulted in only a single or a few spikes of $[Ca^{2+}]_i$ (data not shown).

3.2. *Effects of PKC activators and inhibitors on ATP-induced $[Ca^{2+}]_i$ oscillations*

In the presence of 100 nM PDBu, a phorbol ester which activates PKC, stimulation with 5 μM ATP produced only a single spike of $[Ca^{2+}]_i$ in 73 of 89 cells with no oscillations observed (Fig. 3A). Similar single spikes of $[Ca^{2+}]_i$ following stimulation with ATP were also observed in the presence of another phorbol ester, PMA (50 nM), whereas the inactive phorbol ester 4α-PDD (100 nM) had no effect on the ATP-induced $[Ca^{2+}]_i$ oscillations (data not shown).

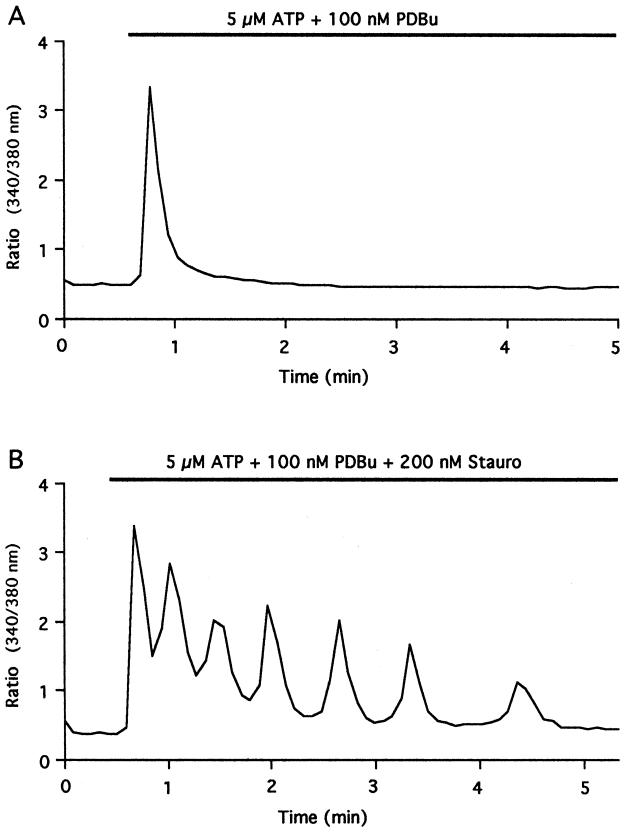

Fig. 3. Effect of the phorbol ester PDBu on ATP-induced $[Ca^{2+}]_i$ oscillations in the absence (A) and presence (B) of staurosporine. (A) Changes in $[Ca^{2+}]_i$ in a single cell exposed simultaneously to 5 μM ATP and 100 nM PDBu. (B) Changes in $[Ca^{2+}]_i$ in a single cell exposed simultaneously to 5 μM ATP and 100 nM PDBu in the presence of 200 nM staurosporine (Stauro). The agents were applied during the period indicated by the horizontal bars.

In order to show that the inhibition of the ATP-induced $[Ca^{2+}]_i$ signal by phorbol esters was due to activation of PKC, we examined if PKC inhibitors can reverse the inhibitory action of PDBu. When the cells were exposed to 5 μM ATP in the presence of both 100 nM PDBu and 200 nM staurosporine, a potent PKC inhibitor, oscillatory changes in $[Ca^{2+}]_i$ occurred in 92 out of 116 cells examined (Fig. 3B). Chelerythrine chloride (10 μM), a more specific PKC inhibitor [21], also reversed the inhibitory effect of PDBu on the ATP-induced $[Ca^{2+}]_i$ signal in 22 out of 39 cells (data not shown), although chelerythrine chloride was not so potent as staurosporine. These results suggest that the phorbol esters suppressed the $[Ca^{2+}]_i$ signal through activation of PKC. When staurosporine was added after exposure to both ATP and PDBu, the inhibitory action of PDBu was not clearly reversed by staurosporine (data not shown).

In several cell types, it has been reported that treatment with PKC inhibitors changes the agonist-mediated oscillations in $[Ca^{2+}]_i$ to a sustained, non-oscillatory increase in $[Ca^{2+}]_i$ or delays the rate of the falling phase in the Ca^{2+} spike [5,7,8]. To investigate this possibility in HSY cells, the ATP-induced changes in $[Ca^{2+}]_i$ were monitored in the presence of PKC inhibitors. Preincubation for 5 min with 200 nM staurosporine, which can reverse the effect of PDBu, did not affect the pattern of $[Ca^{2+}]_i$ oscillations induced by 5 μM ATP, and the desensitization of $[Ca^{2+}]_i$ response was not prevented (Fig. 4A). Even when the preincubation period was extended up to 30 min, staurosporine failed to change the

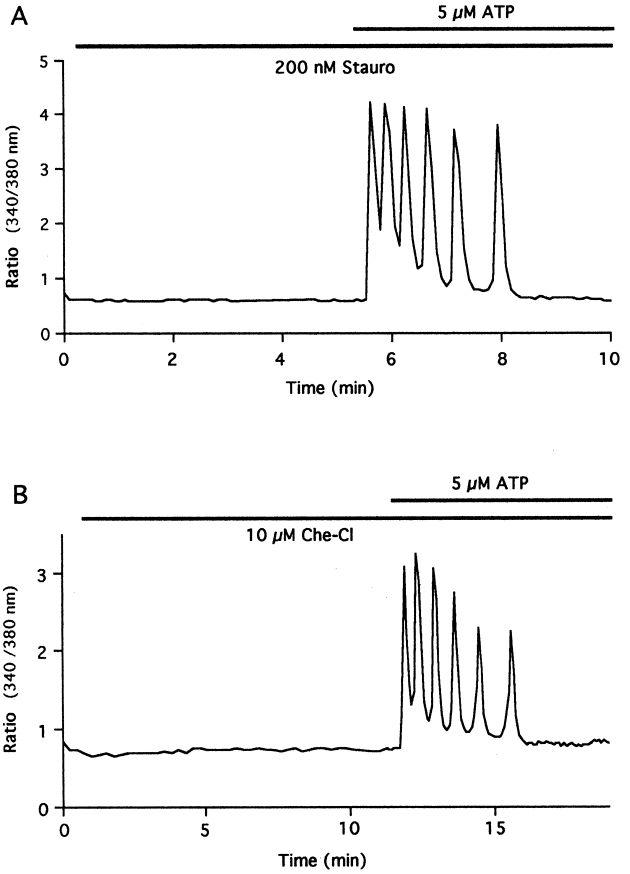

Fig. 4. ATP-induced $[Ca^{2+}]_i$ oscillations in the presence of PKC inhibitors. Cells were stimulated with 5 μM ATP in the presence of 200 nM staurosporine (Stauro) (A) or 10 μM chelerythrine chloride (Che-Cl) (B). ATP and the PKC inhibitors were applied during the period indicated by the horizontal bars.

oscillatory patterns (data not shown). Chelerythrine chloride (10 μM) (Fig. 4B) and another PKC inhibitor, K252a (data not shown), also had no effect on the ATP-induced oscillations in $[Ca^{2+}]_i$.

3.3. Thimerosal-induced $[Ca^{2+}]_i$ oscillations

Sulphydryl reagents including thimerosal have been shown to open the IP_3 receptor channels without activation of phosphoinositide hydrolysis resulting in formation of IP_3 [19,20,22,23]. The IP_3 receptor channels are thought to be sensitized to basal IP_3 levels in the presence of thimerosal. If the thimerosal-induced Ca^{2+} release exhibits oscillatory changes in $[Ca^{2+}]_i$, it would provide further evidence that $[Ca^{2+}]_i$ oscillations may be induced without fluctuations of IP_3 and PKC.

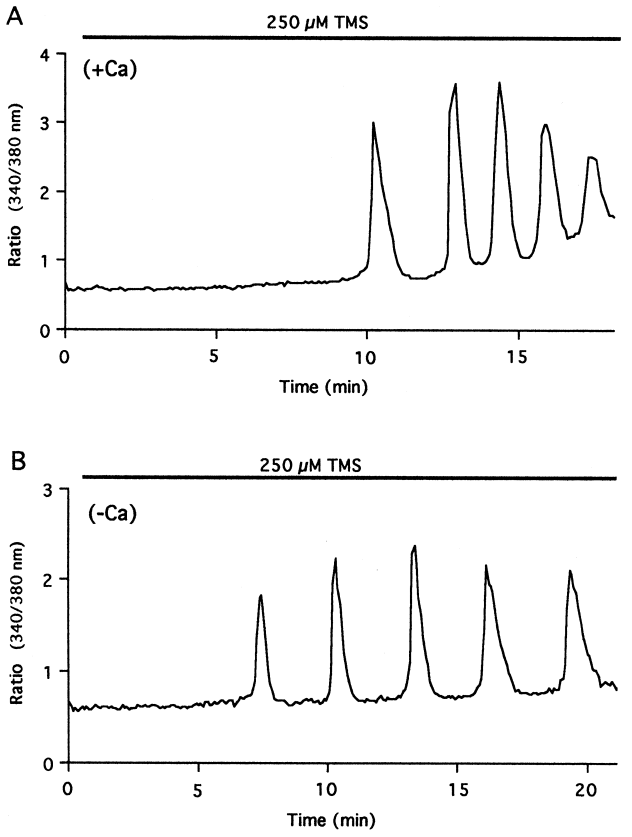

Fig. 5. Thimerosal-induced $[Ca^{2+}]_i$ oscillations in single HSY cells. (A) Changes in $[Ca^{2+}]_i$ in a single cell exposed to 250 μM thimerosal (TMS) in Ca^{2+}-containing solution. (B) Changes in $[Ca^{2+}]_i$ in a single cell exposed to 250 μM TMS in Ca^{2+}-free solution containing 0.2 mM EGTA. TMS was applied during the period indicated by the horizontal bars.

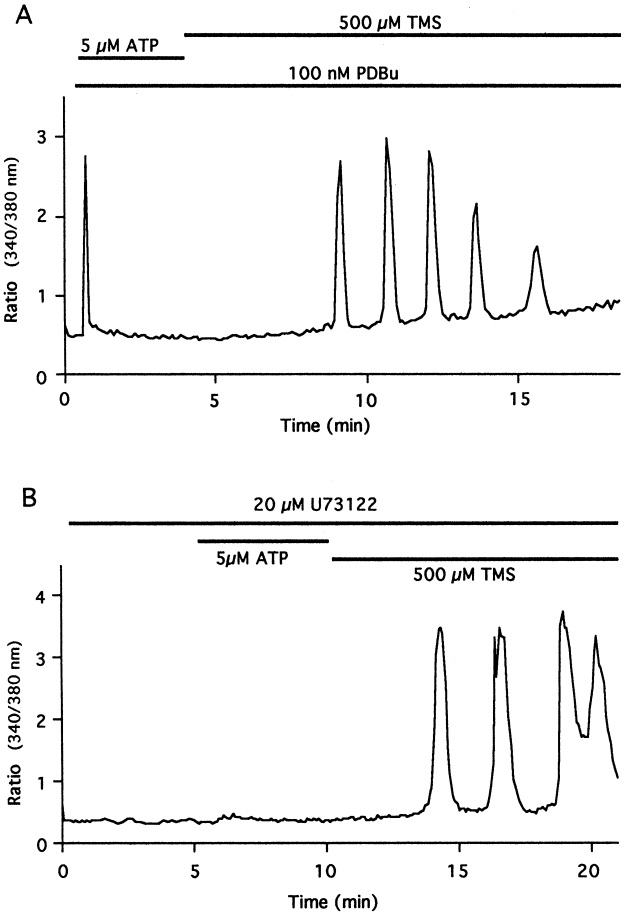

Fig. 6. Effects of PDBu and U73122 on thimerosal-induced $[Ca^{2+}]_i$ oscillations in single HSY cells. The recordings were made in Ca^{2+}-free solution containing 0.2 mM EGTA. (A) In the presence of 100 nM PDBu, cells were stimulated with 5 μM ATP and then exposed to 500 μM thimerosal (TMS). (B) In the presence of 20 μM U73122, cells were stimulated with 5 μM ATP and then exposed to 500 μM TMS. The agents were applied during the period indicated by the horizontal bars.

At concentrations ≥ 200 μM, thimerosal caused repetitive $[Ca^{2+}]_i$ responses following a long latency (5–10 min) before the onset of the rise in $[Ca^{2+}]_i$ (Fig. 5). In the presence of extracellular Ca^{2+}, the changes in $[Ca^{2+}]_i$ were accompanied with a progressive increase in basal $[Ca^{2+}]_i$ (Fig. 5A), probably resulting from the inhibitory effect of thimerosal on the plasma membrane Ca^{2+} pumps [19]. To remove the increases in the basal $[Ca^{2+}]_i$, the thimerosal-induced Ca^{2+} release was monitored in the absence of extracellular Ca^{2+}.

Incubation of the cells with 250 μM thimerosal

caused oscillatory changes in $[Ca^{2+}]_i$ in 118 out of 158 cells and with 500 μM thimerosal in 192 out of 195 cells. Fig. 5B shows a typical pattern of $[Ca^{2+}]_i$ oscillations in a single HSY cell treated with 250 μM thimerosal in Ca^{2+}-free medium. Incubation with 0.5 μM TG, which leads to the leakage of Ca^{2+} from intracellular stores [24], induced a noticeable increase in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+}, but the response did not result in oscillatory changes (data not shown). To show that the thimerosal-induced $[Ca^{2+}]_i$ response is independent of PKC activity, the effect of the PKC activator PDBu on the thimerosal-induced oscillations was examined. Preincubation with 100 nM PDBu did not inhibit the oscillatory changes of $[Ca^{2+}]_i$, while stimulation with ATP resulted in only a single spike in the presence of PDBu (Fig. 6A). To confirm that the thimerosal-induced Ca^{2+} release does not require activation of phosphoinositide hydrolysis, the effect of U73122, a potent inhibitor of phospholipase C, on the thimerosal-induced changes in $[Ca^{2+}]_i$ was examined. As shown in Fig. 6B, preincubation with 20 μM U73122 did not suppress the $[Ca^{2+}]_i$ responses evoked by 500 μM thimerosal, while the ATP-induced increases in $[Ca^{2+}]_i$ were completely inhibited. The inactive analogue, U73443, had no effect on the ATP-induced $[Ca^{2+}]_i$ oscillatory response (data not shown).

4. Discussion

The present study characterized the sinusoidal $[Ca^{2+}]_i$ oscillations in HSY cells induced by ATP. As removal of external Ca^{2+} had little or no effect on the pattern of $[Ca^{2+}]_i$ oscillations, it is evident that the $[Ca^{2+}]_i$ oscillations are due to the periodic release and uptake of Ca^{2+} by intracellular stores. Incubation with U73122 completely inhibited the $[Ca^{2+}]_i$ response to ATP, suggesting that the ATP-induced Ca^{2+} release in HSY cells is mediated by IP_3 produced through activation of phosphoinositide hydrolysis. Phorbol esters changed the ATP-induced oscillations to a single spike. As shown in a variety of cell types [25–30], this effect is probably due to an inhibition of phosphoinositide metabolism through activation of PKC, and a possible explanation for the mechanism of the $[Ca^{2+}]_i$ oscillations is that negative feedback by PKC plays a direct role in the regulation of the ATP-induced $[Ca^{2+}]_i$ oscillations. However, pharmacological inhibition of PKC by PKC inhibitors did not affect the pattern of the ATP-induced $[Ca^{2+}]_i$ oscillations. In addition, the desensitization of the $[Ca^{2+}]_i$ signal during prolonged stimulation was not prevented by the PKC inhibitors. Since the inhibitory effect of the phorbol ester PDBu on the ATP-induced $[Ca^{2+}]_i$ signal was reversed by the PKC inhibitors, it is unlikely that the inhibitors used here were not able to inhibit PKC. Thus, we could not provide evidence that the negative feedback mechanism by PKC plays a central role in the regulation of the ATP-induced $[Ca^{2+}]_i$ oscillations in HSY cells.

Activation of phosphoinositide metabolism, in addition to IP_3, yields diacylglycerol which can activate PKC, and we do not exclude the possibility that PKC is involved in the regulation of $[Ca^{2+}]_i$ signals in HSY cells. However, the PKC inhibitors used here did not have any detectable effect on the ATP-induced $[Ca^{2+}]_i$ oscillations, while the inhibitory effect of PDBu on the $[Ca^{2+}]_i$ signal was suppressed by the same inhibitors. This result would imply that in HSY cells the activation of PKC by agonist stimulation is much more moderate than the direct activation of PKC by the phorbol esters. The role of PKC in the control of the $[Ca^{2+}]_i$ signal in HSY cells remains to be fully explained.

The present study has also demonstrated that thimerosal has the ability to induce oscillatory Ca^{2+} release from intracellular Ca^{2+} stores in HSY cells. Some studies have indicated that thimerosal inhibits the Ca^{2+} pumps of intracellular Ca^{2+} stores [19,31]. Treatment with TG, which results in a release of Ca^{2+} by inhibiting the Ca^{2+} pumps, did not cause $[Ca^{2+}]_i$ oscillations, and this excludes the possibility that the mechanism causing the thimerosal-induced oscillations is associated with inhibition of the Ca^{2+} pumps. It may be possible that feedback regulation of phospholipase C by the released Ca^{2+} itself causes periodic formation of IP_3 resulting in oscillatory changes in $[Ca^{2+}]_i$. However, the findings that the thimerosal-induced Ca^{2+} release was unaffected by treatment with PDBu and U73122 suggest that the activation of phosphoinositide hydrolysis is unlikely to be linked to the action of thimerosal.

The effect of thimerosal on Ca^{2+} release suggests

that the oscillatory changes in $[Ca^{2+}]_i$ can be generated without activation of phosphoinositide metabolism. If the thimerosal- and ATP-induced $[Ca^{2+}]_i$ oscillations are regulated by the same mechanism, our data support the hypothesis that the agonist-stimulated $[Ca^{2+}]_i$ oscillations in HSY cells do not require fluctuations in IP_3 and PKC. However, there are differences in the property and shape of the thimerosal- and ATP-induced $[Ca^{2+}]_i$ oscillations. The thimerosal-induced $[Ca^{2+}]_i$ oscillations appeared as baseline spikes, while the pattern of the ATP-induced $[Ca^{2+}]_i$ oscillations was usually sinusoidal. Putney [32] suggests that the negative feedback by PKC is involved in the regulation of sinusoidal $[Ca^{2+}]_i$ oscillations but not in the regulation of baseline spikes, and it is not excluded that the different types of $[Ca^{2+}]_i$ oscillations may reflect the different mechanisms.

It is established that the activity of IP_3 receptors is enhanced or inhibited by low or high concentrations of Ca^{2+}, respectively [33,34]. Based on the biphasic effects of Ca^{2+} itself on IP_3 receptors, it has been proposed that $[Ca^{2+}]_i$ oscillations are regulated by the positive and negative feedback effects of the released Ca^{2+} on the activity of IP_3 receptors, and this may explain the mechanism of $[Ca^{2+}]_i$ oscillations in HSY cells, as proposed by Tanimura and Turner [15]. Tanimura and Turner [15] demonstrated that exposure of saponin-permeabilized HSY cells to externally applied IP_3 results in $[Ca^{2+}]_i$ oscillations, and this supports the positive and negative feedback mechanism by Ca^{2+} itself to generate the $[Ca^{2+}]_i$ oscillations.

Some investigators have found that intracellular application of IP_3 and stable IP_3 analogues can mimic agonist-activated $[Ca^{2+}]_i$ oscillations [35–38], and these studies could lead to the conclusion that oscillations in $[Ca^{2+}]_i$ occur even when the concentration of IP_3 is constant. In lacrimal acinar cells, however, the intracellular application of IP_3 caused a sustained, nonoscillatory increase in $[Ca^{2+}]_i$, while agonist stimulation resulted in $[Ca^{2+}]_i$ oscillations [7]. In addition, a more recent study showed fluctuations in IP_3 concentration synchronous with $[Ca^{2+}]_i$ oscillations by monitoring the changes in IP_3 concentration using the green fluorescent protein-tagged IP_3 probe [39]. Thus, there is notable disagreement in the experimental evidence for the relationship between $[Ca^{2+}]_i$ oscillations and IP_3 level. The actual mechanisms regulating $[Ca^{2+}]_i$ oscillations may vary in different cell types, and it is likely that more than one mechanism can produce the characteristic patterns of $[Ca^{2+}]_i$ oscillations.

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